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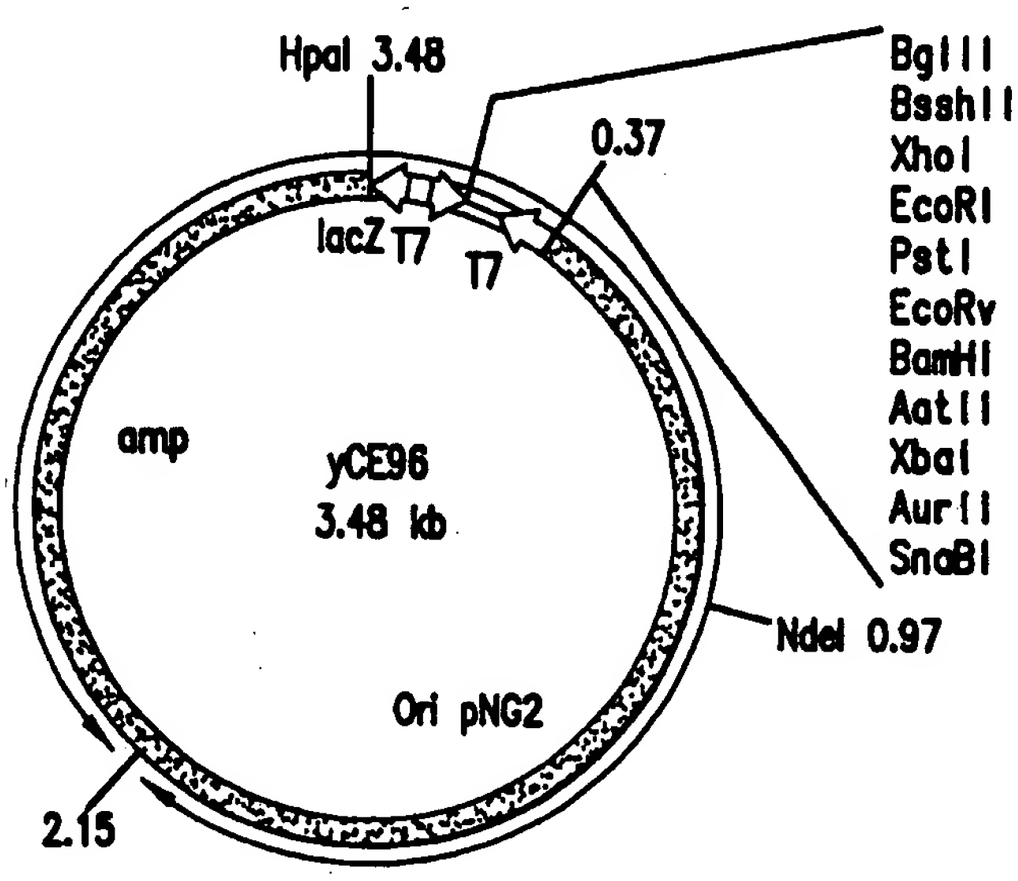

(57) Abstract

The present invention describes a new shuttle vector permitting the expression of engineered toxin mutants and toxin fusion proteins in Corynebacterium. In addition the invention provides a mutant *Pichia pastoris*, a method for producing this mutant and a method of expressing engineered toxin mutants and toxin fusion proteins in the mutant form of *Pichia pastoris*. The invention further provides a mutant Chinese hamster ovary (CHO) cell, a mutant insect cell, a method for producing these mutants and a method of expressing engineered toxin mutants and toxin fusion proteins in the mutant cells.

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NOVEL VECTORS AND EXPRESSION METHODS FOR PRODUCING MUTANT PROTEINS

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates to novel expression systems and vectors for engineered immunotoxins. More specifically the invention relates to a shuttle vector for *E. coli* and *Corynebacteria*. The invention also relates to a method of expressing engineered toxin mutants and toxin fusion proteins in a mutant form of Pichia pastoris, a mutant form Chinese hamster ovary cells, and mutant insect cells and a methods for producing these mutants.

Background Art

U.S. Patent No. 5,167,956 describes *in vivo* T cell killing of 3 logs by immunotoxin anti-CD3-CRM9 or derivatives. This patent also describes the treatment of graft versus host disease, autoimmune disease and T cell leukemia.

A shuttle vector constructed for use in *Corynebacterium* and *E. coli* must contain a replication region (oriR) and a selectable marker that function in both host bacteria, or contain an oriR functional in *E. coli* and an chromosomal integrative mechanism functional in *Corynebacterium*. A naturally occurring plasmid, pNG2, isolated from an erythromycin-resistant Corynebacterium strain fulfills the former criteria (3). However this vector is large (14.4 kb), which reduces its

transformation frequency, which is additionally severely compromised by restriction incompatibilities between *Corynebacterium* and *E. coli* DNA (4). In addition multiple cloning sites are not present in pNG2 which are required to facilitate splicing inserts of toxin and fusion protein toxin genes into the vector.

Thus, the invention meets an important need for an *E. coli* and *Corynebacteria* shuttle vector with multiple cloning sites.

SUMMARY OF THE INVENTION

The present invention describes a new shuttle vector permitting the expression of engineered toxin mutants and toxin fusion proteins in Corynebacterium. In addition the invention provides a mutant *Pichia pastoris*, a method for producing this mutant and a method of expressing engineered toxin mutants and toxin fusion proteins in the mutant form of *Pichia pastoris*. The invention further provides a mutant Chinese hamster ovary (CHO) cell, a method for producing this mutant and a method of expressing engineered toxin mutants and toxin fusion proteins in the mutant CHO cells. These three systems have distinct advantages over *E. coli* expression systems because of their higher yields of secretion into the media compared to *E. coli* (1) thus eliminating the need for refolding procedures from insoluble aggregates (2). Refolding procedures employing denaturing agents although somewhat successful for single chain fusion proteins will not correctly refold divalent single chain fusion proteins

due to their greater complexity. Because divalent immunotoxins are necessary for successful *in vivo* clinical application, the present expression system for engineered immunotoxins is the key to producing a successful immunotoxin.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 depicts the method for producing an *E. coli/Corynebacterium* shuttle vector from the plasmid pNG2.

Fig. 2 depicts the new *E. coli/ Corynebacterium* shuttle vector, yCE96, cotaining nucleotides 1-3476 as shown in SEQ ID NO:1. Residues from positions 1 to 373 and 2153 to 3476 are from the vector LITMUS 29 and contain the polycloning linker sites and the ampicillin resistance marker respectively. Residues from positions 374 to 2152 were the origin sequences from the plasmid pNG2.

Fig. 3a depicts a single chain divalent antibody-mutant-toxin fusion protein produced in Corynebacterium. The toxin is CRM9 and is preceded by the CRM9 promoter and signal sequence. VL and VH linked by a spacer described in U.S. Serial No. 08/739,703, herein incorporated by reference, are from UCHT1. μCH3 and μCH2 are from human IgM. The fusion protein forms the disulfide dimer from the cysteine between the CH2 and CH3 domains during or shortly after secretion. The gene for this fusion protein is constructed by PCR overlap extension to avoid cloning CRM9 until its toxicity is further reduced by a second genetic event, in this case an

additional carboxy terminal protein domain (NIH guidelines, see reference 20).

Fig. 3b shows a double mutant of DT containing the S525F mutation of CRM9 plus an additional replacement within the 514-525 exposed binding site loop to introduce a cysteine coupling site for example T521C can be produced in Corynebacterium ulcerans preceded by the CRM9 promoter and signal sequence. Residue numbering is based on the sequence provided by Shen et al. (17). The double mutant is made in *Corynebacterium ulcerans* by a recombination event between the plasmid producing CRM9-antibody fusion protein and PCR generated mutant DNA with a stop codon at 526. This CRM9-C's can be used to form specific thioether mutant toxin divalent antibody constructs by adding excess bismaleimidohexane to CRM9-C's and coupling to single chain divalent antibody containing a free cysteine at either the end of the μCH4 domain or the μCH3 domain.

DETAILED DESCRIPTION OF THE INVENTION

Provided is an *E. coli/Corynebacterium* shuttle vector comprising the origin of replication of shuttle vector pNG2, polycloning linker sites, and an antibiotic resistance marker with a size less than 4 kb. There are multiple possibilities for the polylinker cloning site and antibiotic resistance marker, which can be selected from those known or later developed. An example of the vector described above is yCE96. The sequence of yCE96 is given below.

The vector can further comprise an insert consisting of a protein-encoding nucleic acid. The encoded protein can contain a disulfide bond. For example, the protein-encoding nucleic acid can encode the binding site mutant DT toxin, CRM9. Alternatively, the protein-encoding nucleic acid can encode a CRM9 further comprising a second attenuating mutation. The second attenuating mutation can be the insertion of a COOH terminal protein domain. The second attenuating mutation can be a COOH terminal mutation that reduces binding activity, but not translocating activity. The second attenuating mutation can be selected from the group consisting of S508F, Y514A/C , K516A/C, V523A/C, N524A/C, K526A/C and F530A/C.

A protein encoding nucleic acid construct of the invention can include a mutation that introduces a cysteine residue into CRM9 or its derivatives. For example, the mutation can be selected from the group consisting of K530C, K516C, D519C and S535C.

A protein encoding nucleic acid construct of the invention can include a mutation that introduces residues or the replacement of residues in CRM9 or its derivatives, to attenuate the blocking effects of anti-diphtheria toxin antibodies. Examples of these mutations are described in the related applications.

A vector of the invention can further comprise the CRM9 iron-independent promoter, wherein the protein-encoding nucleic acid encodes a binding site mutant of diphtheria toxin, and the protein-encoding nucleic acid is

under the control of the CRM9 iron-independent promoter and is preceded by the CRM9 signal sequence.

A method of expressing a diphtheria toxin moiety or other protein is provided. The expression method comprises transfecting a *Corynebacterium ulcerans* or a *Corynebacterium diphtheriae* cell with a vector of the invention under conditions that permit expression of the protein-encoding nucleic acid. The conditions required for expression are the same as those previously used or described herein, including limited iron in the medium.

A method of making a vector of the invention is provided. For example the method comprises a) deleting COOH terminal base pairs of the attenuated CRM9 toxin-encoding nucleic acid using the restriction site Sph I at the toxin nucleotide position 1523 and a restriction site used to clone the COOH terminal part of the toxin into the polylinker cloning sites of yCE96, to produce a gapped, linear, plasmid deleted in the COOH terminal coding region; b) amplifying a product that corresponds to the COOH terminal region of CRM9 deleted in step a), with a PCR primer that includes the desired mutation and 30-40 base pairs homologous to the down stream and upstream regions adjacent to the deletion; c) purifying the amplified product of step b) on an electrophoretic gel; and d) electroporating the product of step c) into a *Corynebacterium* along with the gapped plasmid of step a), under conditions which permit homologous recombination to occur intracellularly.

A method of mutating a protein-encoding nucleic acid in a vector of the invention is provided. The method can comprise a) deleting a region of the COOH terminus-encoding nucleotides of the protein-encoding nucleic acid using a unique restriction site and a restriction site used to clone the COOH terminal part of the toxin into the polylinker cloning sites of the shuttle vector, to produce a gapped, linear plasmid deleted in the COOH terminal coding region is produced; b) amplifying a product that corresponds to the COOH terminus-encoding region deleted in step a), using a PCR primer that includes the desired mutation and 30-40 base pairs homologous to the downstream and upstream regions adjacent to the deletion; c) purifying the amplified product of step b) on an electrophoretic gel; and d) electroporating the purified product of step c) into a *Corynebacterium* along with the gapped plasmid of step a), under conditions which permit homologous recombination to occur intracellularly.

The *Corynebacterium* used in the methods of making the present vectors or mutating proteins can be *Corynebacterium ulcerans*. Alternatively, the *Corynebacterium* can be a *Corynebacterium diphtheriae*, which has been mutated by chemical mutagenesis to exhibit less DNA restriction. Any number of mutations can accomplish this. The presence of the desired mutation is measured by a reduction in restriction in the organism. For example, this can be determined by measuring the efficiency of transformation, i.e., if number of transformants per μg of vector DNA increases over wild type or other mutants.

A mutant strain of *Pichia pastoris* is provided. The mutant strain comprises a mutation in at least one gene encoding elongation factor 2 (EF2). This the mutation comprises a Gly>Arg replacement at a position two residues to the carboxyl side of the modified histidine residue diphthamide. In this manner, the strain is made resistant to the toxic ADP-ribosylating activity of diphtheria and pseudomonas toxins.

A method of expressing a diphtheria toxin protein moiety or a pseudomonas exotoxin A toxin protein moiety is provided. Such a method of the invention comprises transfecting a mutated *Pichia* cell of the invention with a vector comprising a toxin protein-encoding nucleic acid under conditions that permit expression of the protein-encoding nucleic acid in the cell. The conditions are those used for *Pichia* cells and can be optimized for the particular system.

In an expression method using a mutant *Pichia* strain, the encoded protein is glycosylated in the cell to produce of immunotoxins that are resistant to the blocking effects of anti-diphtheria toxin antibodies on the T cell depleting function of CRM9-containing immunotoxins in hūman patients *in vivo*. There is a consensus sequence for glycosylation (NXS/T), which may be removed or inserted to control glycosylation which occurs in all eukaryotes, e.g., *Pichia*.

A method of expressing a diphtheria toxin or a pseudomonas exotoxin A toxin in Chinese hamster ovary

(CHO) cells, insect cells or other eukaryote cells is provided. The method can comprise first making a mutant strain of cells, comprising a mutation in at least one gene encoding elongation factor 2 (EF2), wherein the mutation comprises a Gly>Arg replacement at a position two residues to the carboxyl side of the modified histidine residue diphthamide, and the strain is resistant to the toxic ADP-ribosylating activity of diphtheria and pseudomonas toxins. This homologous recombination method can be used in any eukaryote, due to the high conservation of diphthamide in ekaryotes. Then the mutant cells are transfected with a vector of a type appropriate for the particular cell used, under conditions that permit expression of protein-encoding nucleic acid in the cells. The conditions can be those used for CHO cells and can be routinely optimized for the particular system and cells used.

As with the *Pichia*-expressed nucleic acids, the CHO method produces a glycosylated protein. This can produce immunotoxins that are resistant the blocking effects of anti-diphtheria toxin antibodies on the T cell depleting function of CRM9-containing immunotoxins in human patients *in vivo*.

EXAMPLES

Example 1

A Corynebacterium/Escherichia coli Shuttle Vector for Gene Expression

In brief, a new shuttle vector is constructed using the oriR of pNG2 and the antibiotic resistance marker and multiple cloning sites of the vector Litmus p29 (New England Bio Labs, Inc. Figure 1 depicts a method of making of a vector according to the invention. The new vector, yCE96, is only 3.4 kb in size and can transform both *E. coli* and *Corynebacterium ulcerans* and, thus, can be used to produce toxins and mutant toxins (5).

Cloning OriR from pNG2 plasmid:

pNG2 plasmid DNA was purified from *E. coli* JM109 strain. The 2.6 kb fragment containing oriR was released from pNG2 plasmid by restriction digestion with EcoRI and ClaI endonuclease. After separation and isolation, the 2.6 kb fragment was cloned into pBR322 vector (4361 bp) by the same two restriction sites EcoRI and ClaI, to form a construct of pBRNG with a size of 6.96 kb. This new vector is of limited use, because of its relatively large size and lack of multiple cloning sites.

Sub cloning pNG2 plasmid OriR to Generate a New Vector:

To generate a functional expression vector in Corynebacterium with a small size and a multiple cloning sites, the oriR DNA fragment, released from pBRNG constructs, is combined with a DNA fragment borrowed from

vector litmus p29. The DNA fragment containing the pNG2 oriR was released from pBRNG vector by endonucleases EcoRI and SnaBI, and decreased in size from 2.6 kb to 1.77 kb by cleavage with these enzymes. It is noted that the sequence of the oriR is published, such that it could be constructed without the pNG2 plasmid. Its nt numbers are 374-2152 in the sequence disclosed for yCE96. The DNA fragment borrowed from litmus 29 vector was released by restriction enzyme SnaBI and DraIII, and contains an ampicillin resistant gene and a multiple cloning sites. The two DNA pieces could not be ligated together, because the sticky end of the EcoRI site was not compatible with the sticky end of DraIII. To turn the sticky end into a blunted end, these DNA fragments were treated with T4 polymerase in the presence of 0.1 mM dNTP.

After digestion with restriction enzymes SnaBI and DraIII, and treatment with T4 polymerase in the presence of 0.1 mM dNTP, the litmus 29 vector DNA was dephosphorylated with alkaline phosphatase to remove the 5' phosphate group. The recombined vector was selected by transformation of the ligation mixture into Novablue *E. coli* cells. Five colonies were picked up. All of them contained a plasmid vector. The restriction digestion patterns of purified DNA from these five colonies demonstrated that four colonies contained the correct vector size of 3.4 kb.

The selection of endonuclease to release the oriR from pBRNG was based on the nucleotide sequence of plasmid pNG2 oriR identified by Messerotti et al.(6). The

replication region of pNG2 is 1854 bp, consists of a single oriR ans one major open reading frame. When digested with SnaBI, 75 nucleotides were deleted from 5' end of the identified 1.85 kb oriR sequence. Therefore, the oriR cloned into the new vector, yCE96, was 75 nucleotides smaller than the previously identified 1.85 kb oriR sequence of plasmid pNG2, that is 1779 bp.

Transformation of *Corynebacterium* and *E. coli* by yCE96

E. coli cells were rendered competent by overnight growth followed by resuspension in LB medium containing 10% PEG 8000, 5% DMSO and 50 mM $MgCL_2$, pH 6.5. The cells were heat shocked in the presence of 20 ng of vector DNA per 10^6 cells. *C. ulcerans* were converted to protoplasts as described (4) and transformed by electroporation of 40μg of vector DNA (4).

Unless they have undergone transformation by yCE96, no colonies of *Corynebacterium ulcerans* or *E. coli* grow up under the selection pressure of the presence of carbenicillin. Many colonies are formed after transformation of *E. coli* cells and *C. ulcerans* with the yCE96 vector on the LB plate with 0.1 mg/ml of carbenicillin. The results demonstrate that yCE96 can stably transform *E. coli* and *C. ulcerans*; thus it is an effective shuttle vector between *E. coli* and *C. ulcerans*.

yCE96 is used to introduce foreign proteins into *C. ulcerans*, for example, diphtheria toxin mutants made by PCR site directed mutagenesis. To this end the CRM9 promoter carrying the iron-insensitive mutation (see U.S.

Serial No. 08/739,703, hereby incorporated by reference), as well as the toxin signal sequence have been cloned from CRM9 chromosomal DNA and precede mutant toxin constructs and mutant toxin single chain antibody fusion proteins. Thus, high level synthesis of these engineered proteins, and their secretion into the medium, is expected. Insertion of tandem repeats of these protein-encoding constructs into yCE96 can also be made to achieve even higher production levels. Several constructs and their uses are detailed in Fig. 3 and Fig. 3 legend.

Example 2

Corynebacteriophage-based Vector

An alternate type of shuttle vector containing the integrative mechanism of Corynebacteriophages can be constructed by cloning the corynephage attachment site (attP) and the integrase gene (int) sites from beta Corynebacteriophages into small *E. coli* vectors such as pUC19) not capable of replicating in Corynebacteriae. Addition of another protein coding sequence such as modified CRM sequences (see below) will permit the integration of these sequences into tox-Corynebacteriae. These sequences can be further modified by excision followed by a gapped plasmid methodology described below using a PCR product to achieve any desired mutation or combination of mutations. An advantage of the integrative vector is that it recombines at high efficiency.

Example 3

Using Shuttle Vector yCE96 to Perform Site-Specific Mutagenesis On Diphtheria Toxin Binding Site Mutants in Corynebacteria

A binding site mutant of full length diphtheria toxin residues 1-535 (16) S525F (17) is further modified for chemical coupling by changing a residue in the binding domain (residues 379-535) to cysteine. Preferred residues are those with exposed solvent areas greater than 38%. These residues are K516, V518, D519, H520, T521, V523, K526, F530, E532, K534 and S535 (17). Of these, K516 and F530 are presently preferred, since they are likely to block any residual binding activity (17). However, maximal coupling of the new cysteine residue will be enhanced by the highest exposed solvent surface and proximity to a positively charged residue (which has the effect of lowering cysteine -SH pKa). These residues are at D519 and S535, so that these are also preferred from the above list of possibilities.

These mutations are accomplished by gapped plasmid PCR mutagenesis (18) using the newly designed *E. coli/C. ulcerans* shuttle vector yCE96 containing either the double mutant DT S508F S525F or a CRM9 COOH terminus fusion protein construct having reduced toxicity due to the COOH terminal added protein domain (19). Both of these constructs follow current NIH guidelines for cloning DT derivatives into *E. coli* (Federal Register, Notices, May 7, 1986), Appendix F-II-B, p. 16971) in that they contain two mutations which both individually diminish

toxicity and, therefore, greatly reduce the chance of introducing a wild type toxin into *E. coli* by a single base pair reversion. This mutation is made by deleting the COOH terminal 52 base pairs of the toxin construct using the restriction site Sph I at the toxin nucleotide position 1523 (16) and the restriction site used to clone the COOH terminal part of the toxin into the polylinker cloning sites of yCE96 (Xba or BamHI for example). Since Sph I, Xba, and BamHI only occur singly within vector yCE96 containing the inserted toxin construct, a gapped, linear plasmid, deleted in the COOH terminal coding region is the result. Using PCR the COOH terminal region of CRM9 is rebuilt introducing the desired mutation and including 30-40 base pairs homologous to the down stream and upstream regions adjacent to the gap. The amplified product is gel purified and electroporated into *C. ulcerans* along with the gapped plasmid (18).

Recombination at the homologous regions occurs intracellularly, accomplishing site specific mutagenesis of DT products within *Corynebacteriae* which are not specifically subject to NIH toxin cloning restrictions (20).

Using methods analogous to those described above, but with different restriction enzymes, mutagenesis can be performed anywhere within the toxin molecule. This would be highly useful for the construction of toxin B chain mutations having full translocating activity that are relatively free from the antitoxin blocking activity variably present in the sera of patient populations

resulting from prior immunizations with diphtheria toxoid. Because of the unique features of the present vector, a virtually unlimited number of mutated proteins can be expressed.

Example 4

Expression of mutant ADP-ribosylating toxins and toxin fusion proteins in an EF2 mutant of Pichia pastoris

The invention provides a system for expressing mutant ADP-ribosylating toxins and toxin fusion proteins in a Pichia pastoris mutant. The presently preferred mutant is one that has been rendered insensitive to these toxins by substituting arginine for glycine at a position two amino acids carboxyl to the modified histidine residue diphthamide (position 701 in the EF2 gene based on the numbering system in *S. cerevisiae*).

This mutation has been performed in *S. cerevisiae* and prevents toxin induced ADP-ribosylation rendering the toxin inactive (7). However, the literature has not described or proposed the use of this mutation to generate mutant cells for toxin production. To date, all DT based mutant toxins generated by site directed mutagenesis have been expressed in *E coli*. To date, toxin mutants generated by the application of mutating reagents which are not site specific have only been produced in *C. diphtheriae* and *E. coli*.

Among the advantages of using this mutant *Pichia* are that for antibody toxin fusion proteins, which must have appropriate folding of critical antibody disulfide bonds, the endoplasmic reticulum compartment of the eukaryote yeast offers a similar oxidizing environment to natural eukaryote antibody producing cells such as hybridomas. *Pichia pastoris* has numerous advantages over *S. cerevisiae* (8), including the observation that, for therapeutic proteins, the glycosylation pattern of *Pichia* is much more similar to that of humans compared to *S. cerevisiae*.

The *Pichia* mutant can be constructed by direct transformation of yeast spheroplasts with a mutating oligonucleotide complimentary to the sense strand of *S. cerevisiae* EF2 in the region of the desired mutation but not at the desired mutation (9). Since homology in this area is very high across phyla (10), this sequence will very likely undergo homologous recombination in *P. pastoris*. One example of such a mutating oligonucleotide has the following sequence:

5-GTT ACT TTA CAT GCC GAT GCT ATC CAC AGA AGA GGT GGT CAA ATC ATC CCA-3 (SEQ ID NO:2)

The arginine substitution has been underlined. This nucleic acid can be modified, for example, by shortening on both ends, but this may result in reduced recombination frequency.

The transformation is done in the presence of 10 μM wild type diphtheria toxin or a binding site mutant DT

toxin, such as CRM9, as a selecting agent (11). Several repeat rounds of selection can be utilized. The final selection is performed by transforming *Pichia* with a toxin-containing construct, which will inhibit growth in any cells which lack toxin-resistance mutation in EF2.

The invention also provides a *Pichia* EF2 toxin-resistant clone, which is free of toxin-encoding constructs, to be used for to express alternative constructs. This cell can be generated by popping out the construct via homologous recombination (12).

Many *Pichia pastoris* strains and *E. coli/Pichia* shuttle vectors are available (Invitrogen Corporation), and can be used in an expression system as described above. This technology will be useful for diphtheria toxin and pseudomonas exotoxin A constructs and fusion immunotoxins based on these toxins. The same mutant toxins and toxin-antibody fusion proteins produced in *Corynebacterium* shown in Fig 2 can also be produced in *P. pastoris*. The genetic constructs differ in that in P. pastoris the promotor is AOXI and the secretion or signal sequence is either PHO1 or @-factor. In addition certain codons unique to Corynebacterium may require changing in P. pastoris, as will some codons specifying potential glycosylation sites which would be active in *Pichia* but inactive in *Corynebacterium*. Alternatively, the introduction of glycosylation sites within the CRM9 gene could be used as a method to block antibodies directed at the toxin in patients with high antitoxin titers secondary to recent immunization with diphtheria toxoid.

Example 5

Expression of mutant ADP-ribosylating toxins and toxin fusion proteins in an EF2 mutant of CHO cells.

CHO cells have certain advantages for the production of fusion proteins used as therapeutic reagents: a) Hamster cells are relatively free from retrovirus contamination, b) Protein glycosylation patterns are relatively similar to that seen in humans, and c) CHO cells generally, respond well to gene amplification systems that increase the yield of proteins introduced through DNA transfection. Although it has been reported that mammalian cells can secrete an ETA based fusion protein without succumbing to ETA induced cytotoxicity via ADP-ribosylation (13), this situation is likely due to the resistance of ETA to proteolytic processing at pH >6.0, a feature not shared by DT. Therefore the production of DT based fusion immunotoxins must utilize a line of CHO cells that has been rendered DT insensitive by mutating EF2 by substituting arginine for glycine at a position two amino acids carboxyl to the modified histidine residue diphthamide (position 717 in the EF2 gene based on the numbering system in CHO cell EF2). Two such cell lines have been reported. RE1.22c (14) and KEE1 (15) were isolated by double rounds of chemical mutagenesis and selection with DT or ETA. These mutants were used to determine the physiologic role of dipthamide, the modified histidine residue whose formation is blocked by this mutation. No mention is made in the art of using these cells to make toxins or immunotoxins. The same procedure can be applied to other eukaryote cell lines expressing

toxin sensitivity, such as insect cell lines. These lines have the advantage of secreting large amounts of foreign proteins introduced through baculovirus (21).

Throughout this application various publications are referenced by numbers within parentheses. Full citations for these publications are as follows. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: The Government of the United States of America, as represented by the Secretary, Department of Health and Human Services

(ii) TITLE OF THE INVENTION: NOVEL VECTORS AND EXPRESSION METHODS FOR PRODUCING MUTANT PROTEINS

(iii) NUMBER OF SEQUENCES: 2

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 05-MAR-1998
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/037,196
(B) FILING DATE: 05-MAR-1997

(viii) ATTORNEY/AGENT INFORMATION:
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(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3476 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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What is claimed is:

1. An *E. coli/Corynebacterium* shuttle vector comprising the origin of replication of shuttle vector pNG2, polycloning linker sites, and an antibiotic resistance marker with a size less than 4 kb.

2. The vector of claim 1, wherein the vector is yCE96.

3. The vector of claim 1, wherein the vector further comprises an insert consisting of a protein-encoding nucleic acid.

4. The vector of claim 3, wherein the encoded protein contains disulfide bonds.

5. The vector of claim 4, wherein the protein-encoding nucleic acid encodes a binding site mutant of diphtheria toxin,

further comprising the CRM9 iron-independent promoter and the CRM9 signal sequence,

wherein the protein-encoding nucleic acid is under the control of the CRM9 iron-independent promoter and is preceded by the CRM9 signal sequence.

6. The vector of claim 5, wherein the protein-encoding nucleic acid encodes CRM9.

7. The vector of claim 6, wherein the CRM9 further comprises a second attenuating mutation.

8. The vector of claim 7, wherein the second attenuating mutation is the insertion of a COOH terminal protein domain.

9. The vector of claim 7, wherein the second attenuating mutation is a COOH terminal mutation that reduces binding activity, but not translocating activity.

10. The vector of claim 7, wherein the second attenuating mutation is selected from the group consisting of S508F, Y514A/C , K516A/C, V523A/C, N524A/C, K526A/C and F530A/C.

11. A method of expressing a diphtheria toxin moiety, comprising transfecting a *Corynebacterium ulcerans* or a *Corynebacterium diphtheriae* cell with the vector of claim 1 under conditions that permit expression of the protein-encoding nucleic acid.

12. A method of making the vector of claim 7, comprising:

a) deleting COOH terminal base pairs of the attenuated CRM9 toxin-encoding nucleic acid using the restriction site Sph I at the toxin nucleotide position 1523 and a restriction site used to clone the COOH terminal part of the toxin into the polylinker cloning sites of yCE96, to produce a gapped, linear, plasmid deleted in the COOH terminal coding region;

b) amplifying a product that corresponds to the COOH terminal region of CRM9 deleted in step a), with a PCR primer that includes the desired mutation and 30-40

base pairs homologous to the down stream and upstream regions adjacent to the deletion;

c) purifying the amplified product of step b) on an electrophoretic gel; and

d) electroporating the product of step c) into a *Corynebacterium* along with the gapped plasmid of step a), under conditions which permit homologous recombination to occur intracellularly.

13. The method of claim 12, wherein the *Corynebacterium* is *Corynebacterium ulcerans*.

14. The method of claim 12, wherein the *Corynebacterium* is *Corynebacterium diphtheriae* mutated by chemical mutagenesis to exhibit less DNA restriction.

15. A method of making the vector of claim 5 comprising:

a) deleting a region of the COOH terminus-encoding nucleotides of the protein-encoding nucleic acid using a unique restriction site and a restriction site used to clone the COOH terminal part of the toxin into the polylinker cloning sites of the shuttle vector, to produce a gapped, linear plasmid deleted in the COOH terminal coding region;

b) amplifying a product that corresponds to the COOH terminus-encoding region deleted in step a), using a PCR primer that includes the desired mutation and 30-40 base pairs homologous to the downstream and upstream regions adjacent to the deletion;

c) purifying the amplified product of step b) on an

electrophoretic gel; and

d) electroporating the purified product of step c) into a *Corynebacterium* along with the gapped plasmid of step a), under conditions which permit homologous recombination to occur intracellularly.

16. The method of claim 15, wherein the *Corynebacterium* is *Corynebacterium ulcerans*.

17. The method of claim 15, wherein the *Corynebacterium* is *Corynebacterium diphtheriae* mutated to exhibit less DNA restriction.

18. The method of claim 15, wherein the protein-encoding nucleic acid encodes a protein containing a disulfide bond.

19. The method of claim 15, wherein the encoded protein is a binding site mutant of diphtheria toxin,

further comprising the CRM9 iron-independent promoter,

wherein the protein-encoding nucleic acid is under the control of the CRM9 iron-independent promoter.

20. The method of claim 19, wherein the mutation is the introduction of a cysteine residue into CRM9 or its derivatives.

21. The method of claim 19, wherein the mutation is selected from the group consisting of K530C, K516C, D519C and S535C.

22. The method of claim 19, further comprising a second attenuating mutation, which is the insertion of a COOH terminal protein domain.

23. The method of claim 19, further comprising a second attenuating mutation, which is a COOH terminal mutation that reduces binding activity, but not translocating activity.

24. The method of claims 19, further comprising a second attenuating mutation selected from the group consisting of S508E, Y514A/C, K516A/C, V523A/C, N524A/C, K526A/C and F530A/C.

25. The method of claim 19, wherein the mutation is the introduction of residues or the replacement of residues in CRM9 or its derivatives, to attenuate the blocking effects of anti-diphtheria toxin antibodies.

26. A mutant strain of *Pichia pastoris*, comprising a mutation in at least one gene encoding elongation factor 2 (EF2),

wherein the mutation comprises a Gly>Arg replacement at a position two residues to the carboxyl side of the modified histidine residue diphthamide, whereby the strain is resistant to the toxic ADP-ribosylating activity of diphtheria and pseudomonas toxins.

27. A method of expressing a diphtheria toxin moiety or a pseudomonas exotoxin A toxin moiety, comprising transfecting the cell of claim 26 with a vector comprising

a toxin protein-encoding nucleic acid under conditions that permit expression of the protein-encoding nucleic acid in the cell.

28. The method of claim 27, wherein the encoded protein is glycosylated in the cell.

29. A method of expressing a diphtheria toxin or a pseudomonas exotoxin A toxin, comprising:

transfecting, with a vector of claim 3, under conditions that permit expression of protein-encoding nucleic acid, a mutant strain of Chinese hamster ovary cells, comprising a mutation in at least one gene encoding elongation factor 2 (EF2),

wherein the mutation comprises a Gly>Arg replacement at a position two residues to the carboxyl side of the modified histidine residue diphthamide,

whereby the strain is resistant to the toxic ADP-ribosylating activity of diphtheria and pseudomonas toxins.

30. The method of claim 29, wherein the encoded protein is glycosylated in the cell.

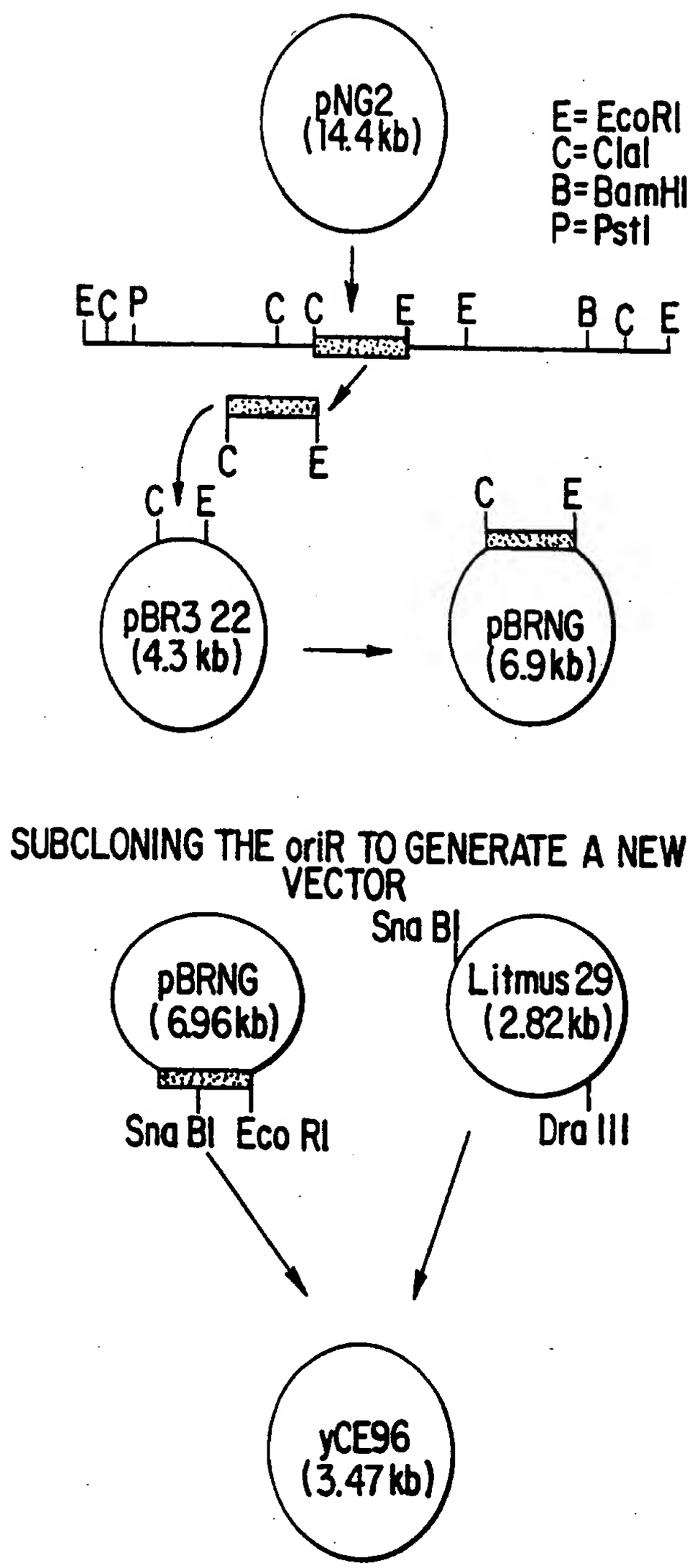
31. A method of expressing a diphtheria toxin or a pseudomonas exotoxin A toxin, comprising:

transfecting, with a vector of claim 3, under conditions that permit expression of protein-encoding nucleic acid, a mutant strain of insect cells, comprising a mutation in at least one gene encoding elongation factor 2 (EF2),

wherein the mutation comprises a Gly>Arg replacement at a position two residues to the carboxyl side of the modified histidine residue diphthamide,

whereby the strain is resistant to the toxic ADP-ribosylating activity of diphtheria and pseudomonas toxins.

32. The method of claim 31, wherein the encoded protein is glycosylated in the cell.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C= ClaI
B= BamHI
P= PstI
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C C
E E
B C E
C E
C E
pBR3 22
(4.3 kb)
C E
pBRNG
(6.9kb)
SUBCLONING THE oriR TO GENERATE A NEW VECTOR
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Litmus 29
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Dra III
yCE96
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FIG. 1

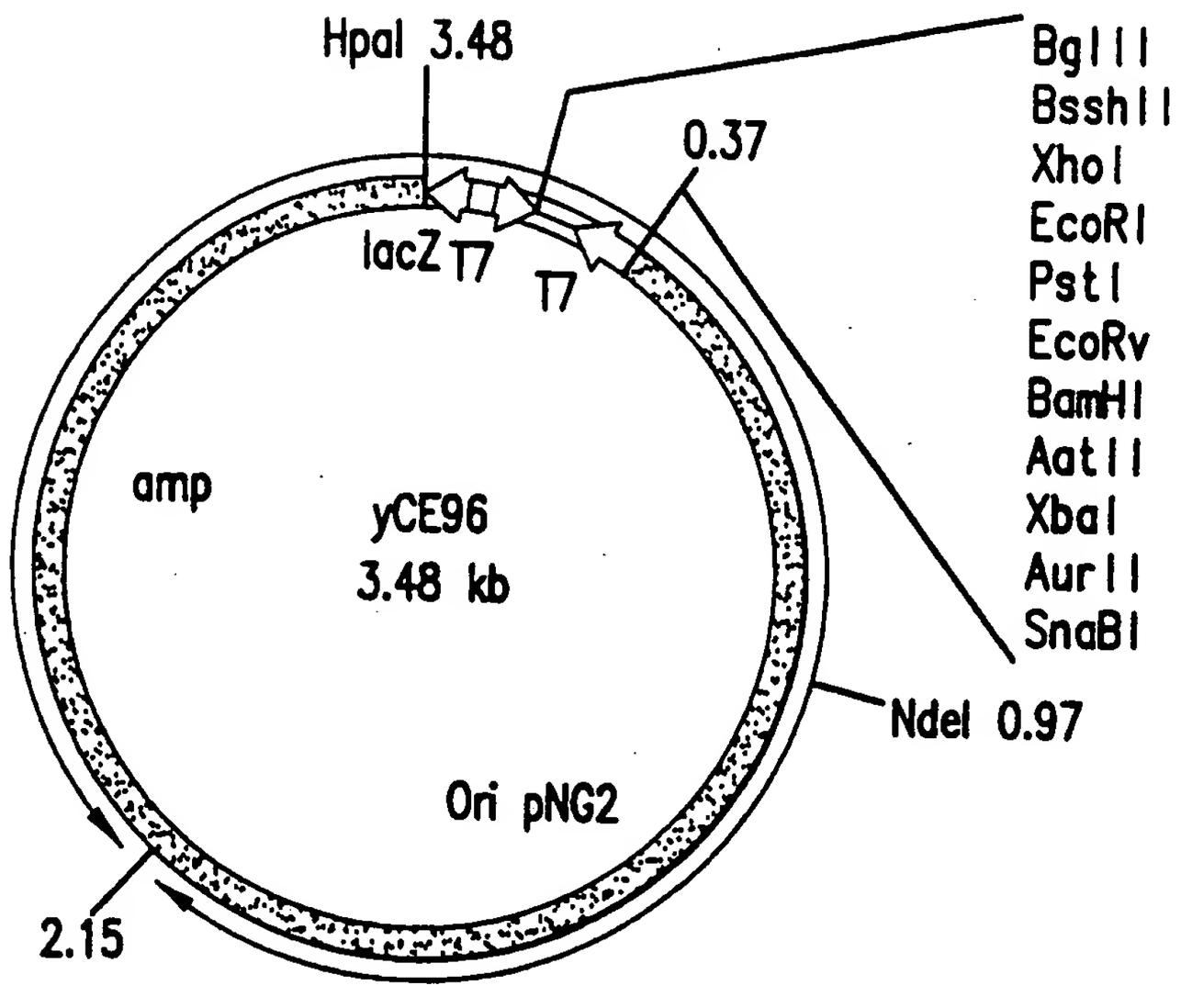
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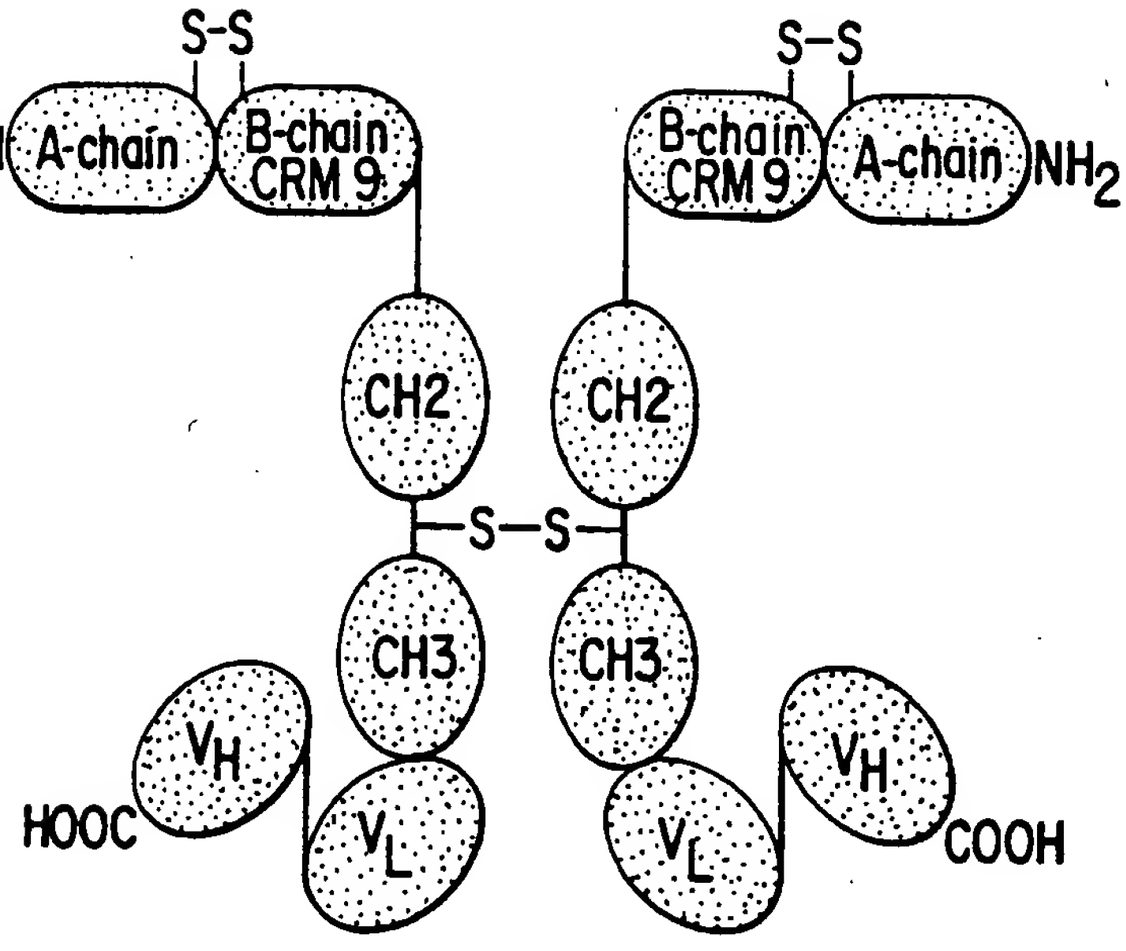
FIG.2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CH3
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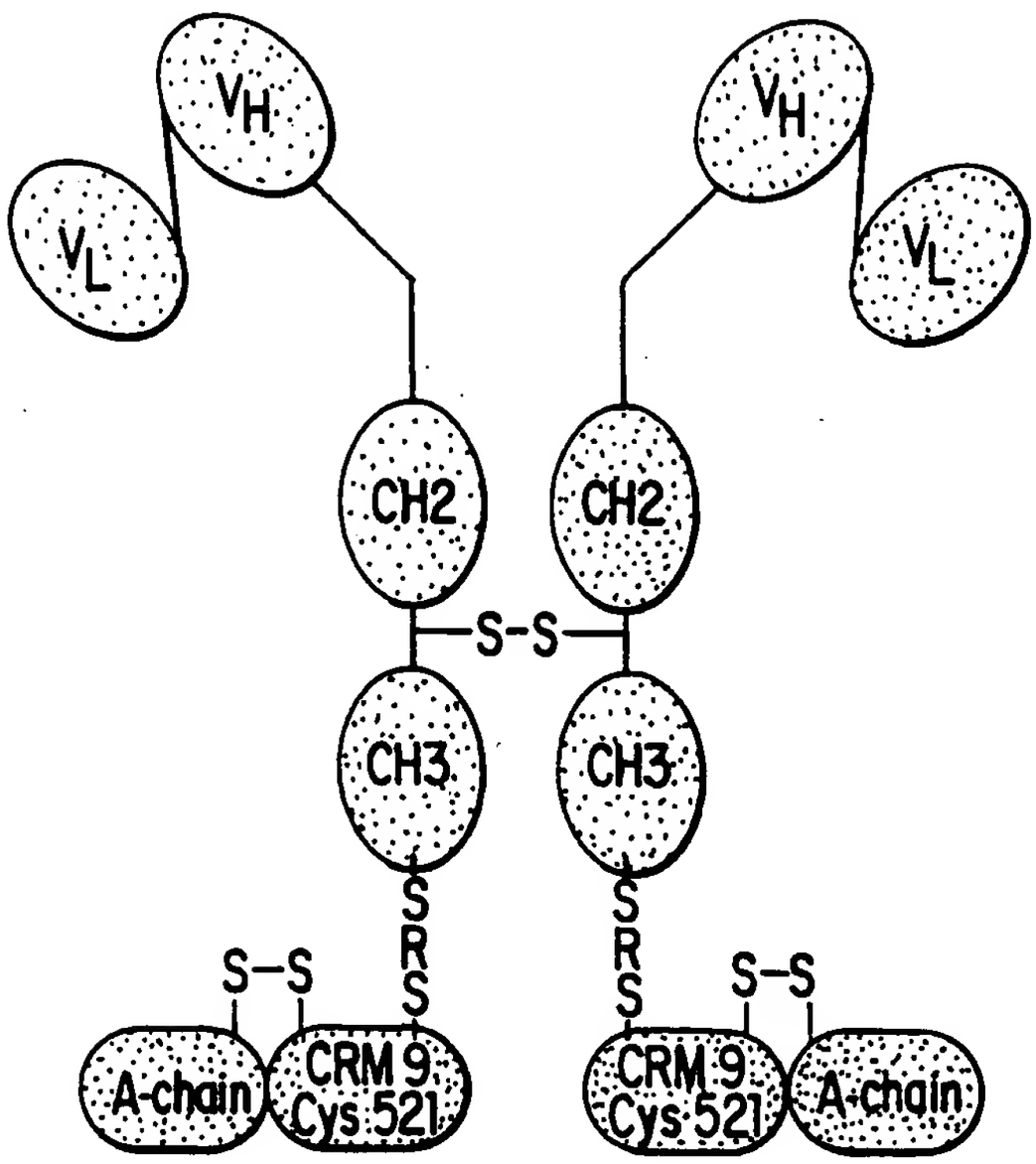
VL
VL
VH
HOOC
COOH

FIG. 3a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S-S
A-chain
CRM 9
Cys 521
CRM 9
Cys 521
A-chain

FIG. 3b